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13. ABSTRACT (Maximum 200 Words)

The toxicity of chemically reactive oxygen species (ROS) is thought to make a significant contribution to the death of nerve cells caused by many neurotoxins as well as in stroke and Parkinson's disease. During all of these events, some groups of nerve cells are spared relative to others. It is therefore likely that biochemical mechanisms exist which lead to increased resistance to oxidative stress and other forms of cytotoxicity. It was the goal of this proposal to understand how nerve cells defend themselves against neurotoxins that kill cells via ROS and oxidative stress. To accomplish this goal, we have studied toxin sensitive cell death pathways and have selected groups of cells which are very resistant to ROS generated in a model system which mimics some aspects of acute neurotoxicity and stroke. It was shown that the translation factor eIF2α mediates cell death involving oxidative stress, while the classical pro-apoptosis factor Bax is not involved. The activation of soluble guanylate cyclase is also required for cell death, for dopamine D4 receptor activation inhibits cGMP production and blocks cell death. Other inhibitors of oxidative stress induced nerve cell death were also discovered. These include a unique group of plant flavanoids and the activation of the transcription factor HIF-1. Finally, it was shown that while there is a great deal of cross-resistance to many neurotoxins, the components of the cell death pathways are sometimes distinct.

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Table of Contents

INTRODUCTION

The accumulation of oxidatively damaged macromolecules results from the exposure to several chemical warfare agents and is thought to contribute heavily toward the genesis of numerous diseases of old age. To date, a great deal is known about the causes of oxidative stress, but very little about how cellular metabolism is altered to successfully cope with this condition. If it were possible to understand at the molecular level how cells are able to cope with increases in oxidative stress, then it should be possible to artificially modify these defense mechanisms to inhibit or slow the damage. The research accomplishments outlined below help to define the molecular mechanisms by which cells become resistant to oxidative stress and also identify a number of conditions which block nerve cell death pathways. This knowledge may be used to slow down or prevent oxidative damage associated with exposure to neurotoxins as well as those associated with the diseases of aging. The information should have a significant impact upon the treatment of neurotoxicity and environmental conditions associated with oxidative stress.

BODY

As outlined in the abstract, several areas of work have been completed during the last three years. These include examining the role of the cell death (apoptosis) gene in oxidative glutamate toxicity and excitotoxicity, the role of eIF2α in oxidative stress, the involvement of oxidative glutamate toxicity in excitotoxicity, the role of soluble guanylate cyclase in neuroprotection via the activation of dopamine receptors, the neuroprotective mechanisms of flavonoids, the overlap of neuroprotective pathways, and finally, the role of the transcription factor HIF-1 in neuroprotection. The results from each of these studies will be outlined below and the completed manuscripts with all of the data and details are affixed to the appendix.

A. Bax

Bax is a required protein for most forms of apoptotic programmed cell death. It is thought to regulate the permeability of mitochondria to proteins which mediate the activation of caspases and other components of apoptosis. Nerve cell death from both oxidative glutamate toxicity and excitotoxicity share a few characteristics with classical apoptosis, but no one has ever examined the role of Bax, the major player in the classical apoptosis pathway, in these pathways. This is critical information, for if Bax is not involved, then another form of programmed cell death than classical apoptosis must take place during glutamate neurotoxicity. To approach this issue, we used mice which lack the Bax gene, so called Bax knock-out mice. These mice are developmentally abnormal and die before birth, but it is possible to obtain brain nerve cells for primary cultures from E14 embryos and study their response to glutamate. When this was done, it was shown that cortical neurons died equally well when they were isolated from homozygous or heterozygous Bax deletions and from wild type mice. In contrast, the rate of spontaneous cell death when cells are initially placed in culture, due to growth factor withdrawal, was greatly retarded in the cell cultures from Bax knock-out animals. These results clearly show that Bax is not involved in either oxidative glutamate toxicity or excitotoxicity caused by a brief exposure to low concentrations of glutamate. They therefore strongly suggest that a unique form of nerve cell death is involved in glutamate toxicity, which certainly utilizes a distinct set of molecular components.

B. eIF2α and oxidative stress

Although programmed cell death (PCD) is a widely used mechanism for sculpturing the developing nervous system, its inappropriate activation leads to premature nerve cell death in neuropathological disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). These forms of nerve cell death as well as those caused by a wide variety of neurotoxins are thought to be linked to oxidative stress, for antioxidant systems are upregulated and there is extensive evidence for excessive lipid and protein peroxidation. Associated with oxidative stress, there is usually an early and highly specific decrease in neuronal glutathione content. In the substantia nigra of PD patients, this loss may precede the death of dopaminergic neurons. In

addition, the inhibition of γ-glutamyl-cysteine synthetase (γGCS), the rate limiting step in GSH synthesis, results in the selective degeneration of dopaminergic neurons, and also potentiates the toxicity of 6 hydroxydopamine, MPTP and MPP^+. These data suggest that GSH and oxidative stress play pivotal roles in neurotoxicity and in the pathogenesis of AD and PD.

There are several ways in which the concentration of intracellular GSH and the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non-receptor mediated pathway which involves the glutamate-cystine antiporter, system Xc^-. Under normal circumstances the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc^- antiporter in exchange for intracellular glutamate. Cystine is ultimately converted to cysteine and utilized for protein synthesis and to make the antioxidant glutathione (GSH). However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is inhibited, and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of events which include the depletion of GSH, a requirement for macromolecular synthesis and caspase activity, lipoxygenase (LOX) activation, soluble guanylate cyclase activation, reactive oxygen species (ROS) accumulation, and finally Ca^{2+} influx.

Programmed cell death caused by oxidative glutamate toxicity has characteristics of both apoptosis and necrosis, and has been well studied in primary neuronal cell cultures, neuronal cell lines, tissue slices, and in the immortalized mouse hippocampal cell line, HT22. HT22 cells lack ionotropic glutamate receptors but die within 24 hours after exposure to 1-5 mM glutamate. Although the biochemical events have been well studied, little has been done to identify the transcriptional/translational changes which contribute to the glutamate-induced pathway of programmed cell death. Changes in gene expression clearly play a role in the cell death cascade since macromolecular synthesis is required early in the death pathway.

Using an experimental nerve cell model for oxidative stress and an expression cloning strategy, a gene involved in oxidative stress-induced programmed cell death was identified which both mediates the cell death program and regulates GSH levels. Two stress-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor eIF2α and express a low amount of eIF2α. Sensitivity is restored when the clones are transfected with full length eIF2α; transfection of wild-type cells with the truncated eIF2α gene confers resistance. The phosphorylation of eIF2α also results in resistance to oxidative stress. In wild-type cells oxidative stress results in rapid glutathione depletion, a large increase in peroxide levels, and an influx of Ca^{2+}. In contrast, the resistant clones maintain high glutathione levels and show no elevation in peroxides or Ca^{2+} when stressed, and the glutathione synthetic enzyme gamma-glutamyl cysteine synthetase (γGCS) is elevated. The change in γGCS is regulated by a translational mechanism. eIF2α is therefore a critical regulatory factor in the response of nerve cells to oxidative stress and in the control of the major intracellular antioxidant, GSH, and may play a central role in the many neurodegenerative diseases and toxicities associated with oxidative stress.

C. Oxidative glutamate toxicity and excitotoxicity

The physiological consequences of extracellular glutamate are mediated by three classes of membrane proteins within the central nervous system (CNS). These are ionotropic glutamate receptors, metabotropic glutamate receptors, and the cystine/glutamate antiporter. Ionotropic glutamate receptors have two known roles. They are responsible for the majority of excitatory neurotransmission within the CNS and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors. This phenomenon, which can be reproduced in cell culture is termed excitotoxicity. In contrast to ionotropic glutamate receptors, the metabotropic glutamate receptors (mGluRs) are G-protein coupled membrane proteins with a wide variety of biological functions. As described above, a third target for extracellular glutamate in the CNS is the

inhibition of the glutamate/cystine antiporter x_c^- which results in a form of oxidative stress and cell death called oxidative glutamate toxicity. The glutamate/cystine antiporter couples the import of cystine to the export of glutamate. Concentrations of extracellular glutamate as low as 100μM, which is well below the level of extracellular glutamate found in models of stroke and trauma, completely inhibit the uptake of cystine. Cystine is required for the synthesis of the potent intracellular reducing agent glutathione (GSH). When GSH is depleted by extracellular glutamate, cells die from a form of programmed cell death.

The potential role of oxidative glutamate toxicity in ischemia and trauma is not understood, but there have been strong indications that several cell death pathways are involved in the excitotoxicity cascade. In localized cerebral infarction, the neurons in the epicenter die rapidly, while those more distal remain viable for several hours. Multiple forms of nerve cell death have also been identified in excitotoxic CNS primary culture paradigms following exposure to glutamate. In primary cultures of cerebellar granule cells exposed to glutamate, there is a rapid necrotic phase, followed by delayed apoptotic-like cell death. During oxygen-glucose deprivation of primary mouse cortical cultures or organotypic cultures of the rat hippocampus, some cell death occurs from non-ionotropic receptor-mediated mechanisms. All of these observations are consistent with in vivo data which show that non-receptor mediated programmed cell death may occur following ischemic insults. In addition, a number of parameters change dramatically during CNS stress which lead to the observed high exogenous glutamate. These include the direct release of glutamate from cells, the enzymatic conversion of glutamine to glutamate, and the shut down of nerve and glial glutamate uptake systems by pro-oxidant conditions. It is therefore of interest to determine if oxidative glutamate toxicity can play a significant role in nerve cell death associated with the excitotoxicity cascade.

In the manuscript attached to the appendix, we show that a portion of the cell death associated with NMDA receptor initiated excitotoxicity can be caused by oxidative glutamate toxicity. In primary mouse cortical neurons cell death resulting from the short term application of 10μM glutamate can be divided into NMDA and non-NMDA receptor dependent phases. The non-NMDA receptor dependent component is associated with high extracellular glutamate and is inhibited by a variety of reagents which uniquely block oxidative glutamate toxicity. These include metabotropic glutamate receptor agonists, antioxidants, and a caspase inhibitors. In addition, it is shown that the concentration of extracellular glutamate rises to several hundred micromolar, probably due to the conversion of glutamine to glutamate in the culture medium (glutamine is equally high in CNS tissue) by the enzyme glutaminase released from lysed cells. These results suggest that oxidative glutamate toxicity toward neurons lacking functional NMDA receptors can be a component of the excitotoxicity initiated cell death pathway.

D. Dopamine and D4 Receptors

The protective effects of dopamine, apomorphine and apocodeine, but not epinephrine and norepinephrine, are antagonized by dopamine D4 antagonists. A dopamine D4 agonist also protects and this protective effect is inhibited by U101958, a dopamine D4 antagonist. Although the protective effects of some of the catecholamines are correlated with their antioxidant activities, there is no correlation between the protective and antioxidant activities of several other ligands. Normally glutamate causes an increase in reactive oxygen species (ROS) and intracellular Ca^{2+}. Apomorphine partially inhibits glutamate-induced ROS production and blocks the opening of cGMP-operated Ca^{2+} channels which lead to Ca^{2+} elevation in the late part of the cell death pathway. These data suggest that the protective effects of apomorphine on oxidative stress-induced cell death are mediated by dopamine D4 receptors via the regulation of cGMP-operated Ca^{2+} channels.

E. Flavonoids

Flavonoids are a group of several hundred diphenylpropanes which are widely distributed in plants and are generally thought to be beneficial dietary supplements, perhaps working as antioxidants. Since we have previously shown that several aromatic antioxidants are able to

shunt. AD brain also has increased enzymatic activities in both pathways relative to age-matched controls. In the clonal and CNS cortical nerve cell culture systems the Aβ-induced changes in glucose metabolism are due to the activation of the transcription factor (HIF-1), which also decreases intracellular reactive oxygen species (ROS). As a result of Aβ induced changes in glucose metabolism, Aβ resistant cells are more readily killed by glucose starvation, deoxyglucose, and by classes of antipsychotic drugs that inhibit glucose uptake. These results suggest that some populations of nerve cells in AD brain may be vulnerable to conditions such as stroke, type II diabetes, and drugs that limit glucose availability or uptake.

Finally, the activation of glial cells by toxins such as Aβ leads to the secretion of toxic cytokines as well as pro-oxidants such as superoxide. Glial activation is associated with most chronic neurodegenerative diseases, such as AD and Parkinson's. We have shown that exposure of glial cells to MIM or DFO prior to the addition of Aβ, which activates glial cells, prevents glial activation, and that this effect is highly correlated with the DNA binding of HIF-1 in gel-shift assays. It follows that the induction of HIF-1 activity by iron chelators facilitates neuroprotection both directly and indirectly by blocking glial activation which can severely stress or kill neurons.

KEY RESEARCH ACCOMPLISHMENTS

1. The Bax gene product is not involved in oxidative glutamate toxicity or excitotoxicity.
2. The translation initiation factor eIF2α can serve as a switch which determines whether nerve cells live or die during oxidative stress. The eIF2α functions by regulating the level of intracellular glutathione by determining the level of the glutathione synthetic enzyme, γGCS.
3. The unique programmed cell death pathway, oxidative glutamate toxicity, is a component of the widely studied but little understood excitotoxicity cascade, which is involved in many forms of oxidative stress induced by trauma and disease.
4. The activation of dopamine D4 receptors protects cells from oxidative stress induced cell death.
5. Flavonoids and related compounds inhibit neurotoxicity by three distinct mechanisms: enhancing GSH levels, acting as antioxidants, blocking cGMP induced calcium entry.
6. Cells selected for resistance to one neurotoxin (glutamate) are resistant to some other neurotoxins.
7. Reagents which protect cells from glutamate toxicity also protect cells from peroxide toxicity, but not from arsenite and cisplatin toxicity.
8. The activation of the transcription factor HIF-1 was shown to protect nerve cells from several forms of oxidative stress via the activation of glycolysis. This is an important, clinically relevant, observation since the synthesis of HIF-1 can be initiated by clinically approved drugs such as iron chelators.

REPORTABLE OUTCOMES

a. Nine manuscripts: previously unsubmitted one in appendix
b. One Ph.D. student (Shirlee Tan) who worked on eIF2α graduated and now works for the EPA
c. Research experience for a pre-med student (Dana Piasecki) working on this project
d. Research experience for post-doctoral students, T. Soucek, Y. Sagara, and K. Ishigi

CONCLUSIONS

During the past three years we have studied one of the major nerve cell death pathways activated in response to various forms of oxidative stress and characterized some of the intermediate steps. More importantly, we have identified a number of reagents and conditions which specifically block the cell death pathway. These neuroprotective agents include dopamine analogues which activate the dopamine D4 receptor, a small class of flavonoids, conditions which cause the phosphorylation of the translation initiation factor eIF2α, and the activation of

the transcription factor HIF-1. This work resulted in seven published manuscripts in very good journals, and two review articles.

On the basis of this information it may then be possible to artificially activate the HIF-1 transcription factors or shut off the activity of eIF2α and therefore express the set of proteins involved in the resistance to oxidative stress. For example, HIF-1 mediates the induction of a battery of genes involved in neuroprotection. Some of these genes are upregulated in the resistant cells described by our laboratory. Since HIF-1 can be induced pharmacologically by iron chelators, it may be possible to induce the necessary antioxidant genes before or immediately after exposure to neurotoxins, leading to protection. Clearly more sophisticated methods will become available and can be easily tested in the experimental system described here. In addition, we have identified a unique set of flavonoids which are neuroprotective, and these should be studied in much greater detail because of their unique modes of action and because, as beneficial natural products, they are not toxic and could immediately be put into clinical trials. The same argument could be made for the protective dopamine D4 receptor agonists, but these could prove difficult clinically because of psychoactive side effects. Finally, the general insight of how cells become resistant to oxidative stress will lead to a better understanding of the cause of free radical damage and its role in aging and the various neuropathologies of old age.

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Personnel receiving pay from the research effort

David R. Schubert, Principal Investigator
Richard Dargusch, Research Assistant
Shirlee Tan, Graduate Research Assistant

APPENDIX

"The Regulation of Glucose Metabolism by HIF-1 Mediates a Neuroprotective Response to Amyloid Beta Peptide"

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The Regulation of Glucose Metabolism by HIF-1 Mediates a Neuroprotective Response to Amyloid Beta Peptide

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Summary

It is frequently argued that both amyloid beta (Aβ) and oxidative stress are involved in the pathogenesis of Alzheimer's disease (AD). We show here that clonal nerve cell lines and primary cortical neurons that are resistant to Aβ toxicity have an enhanced flux of glucose through both the glycolytic pathway and the hexose monophosphate shunt. AD brain also has increased enzymatic activities in both pathways relative to age-matched controls. The Aβ-induced changes in glucose metabolism are due to the activation of the transcription factor hypoxia inducible factor 1 (HIF-1). As a result of Aβ-induced changes in glucose metabolism, Aβ-resistant cells are more readily killed by glucose starvation and by classes of antipsychotic drugs that inhibit glucose uptake.

Introduction

Alzheimer's disease (AD) is characterized by extensive cell death within the brain (Terry et al., 1994). However, as with most other neurobiological diseases, not all of the cells within an afflicted area die. There appear to be populations of cells which survive the same conditions that kill their neighbors. Although there has been intense interest in the mechanisms of cell death, very few studies have attempted to understand how nerve cells become resistant to potential toxins or loss of trophic support. In the case of AD, many believe that the Aβ peptide is the primary toxic agent responsible for nerve cell death (Klein et al., 2001). Therefore, by examining how cells become resistant to Aβ toxicity, one can learn more about the toxicity pathway as well as the mechanisms of amyloid resistance. To address the mechanisms involved in resistance to Aβ toxicity, a series of Aβ-resistant clones were derived from the rat sympathetic nerve-like cell line PC12 and the central nervous system cell line B12 by growth for 4 months in the presence of Aβ- and subsequent cloning (Behl et al., 1994; Sagara et al., 1996). Unlike the parental cells, these resistant clones do not respond to high levels of Aβ by dying or by accumulating peroxides. The resistant cells are also much less sensitive to H_2O_2 and t-butyl-H_2O_2, in part due to the increased expression and activities of the antioxidant enzymes catalase and glutathione peroxidase relative to the parental cells. To combat increases in oxidative stress caused by Aβ, it is necessary for the cell to produce increased amounts of molecules having a high transfer potential such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH). These molecules serve as cofactors for antioxidant enzymes and for maintaining high levels of reduced glutathione (GSH), the cell's major antioxidant. The production of reducing equivalents is best achieved via glycolysis to produce NADH and by the hexose monophosphate shunt (HMS) to produce NADPH. Changes in glucose metabolism are associated with AD (Mielke et al., 1991), and the upregulation of glucose metabolism is tied to the activation of the transcription factor HIF-1 (Semenza, 1999).

HIF-1 is a heterodimeric transcription factor comprised of two subunits, HIF-1α and HIF-1β (Wang et al., 1995). Under normoxic conditions, HIF-1 DNA binding and transcription of hypoxia-inducible genes does not occur. Because oxygen and iron are required for the inhibition of HIF-1 activity, iron chelators can mimic hypoxia and induce HIF-1. HIF-1 mediates the adaptation of cells to hypoxia and hypoglycemia by upregulating the expression of genes involved in erythropoiesis, angiogenesis, glucose transport, and glycolysis (Semenza, 1999). Cells that make more use of glycolysis than oxidative phosphorylation to meet their energy demands tend to lower the mitochondrial production of ROS (Brand, 1997).

Two observations suggested that HIF-1 activity may be involved in protecting cells from Aβ neurotoxicity. Iron chelators and heat shock protect cells from Aβ toxicity (Behl and Schubert, 1993; Schubert and Chevion, 1995), and both conditions induce HIF-1 activity (Katschinski et al., 2002). It was therefore asked if HIF-1 activity is the mechanism used to regulate the Aβ-induced changes in glucose metabolism. The data presented below show that glycolysis, the HMS, and glucose uptake are upregulated in the Aβ-resistant cells as well as in cortical neurons exposed to subtoxic levels of Aβ, and that glycolytic and HMS enzymes are more active in lysates of AD brain compared to age-matched controls. The enhanced glycolysis in the cultured cells is due to the activation of HIF-1 by Aβ, and HIF-1α is more highly expressed in AD transgenic mice. The physiological consequences of enhanced glucose metabolism in neurons are increased sensitivity to both hypoglycemic stress and antipsychotic drugs that inhibit glucose uptake.

Results

Glycolytic Enzymes and Glucose Flux Are Upregulated in Aβ-Resistant Cells

Since Aβ-resistant nerve cells are resistant to multiple forms of oxidative stress (Behl et al., 1994; Sagara et al., 1996), it was asked if they alter their glucose metabolizing pathways to produce a more reductive environment. Glycolysis, the sequential conversion of glucose

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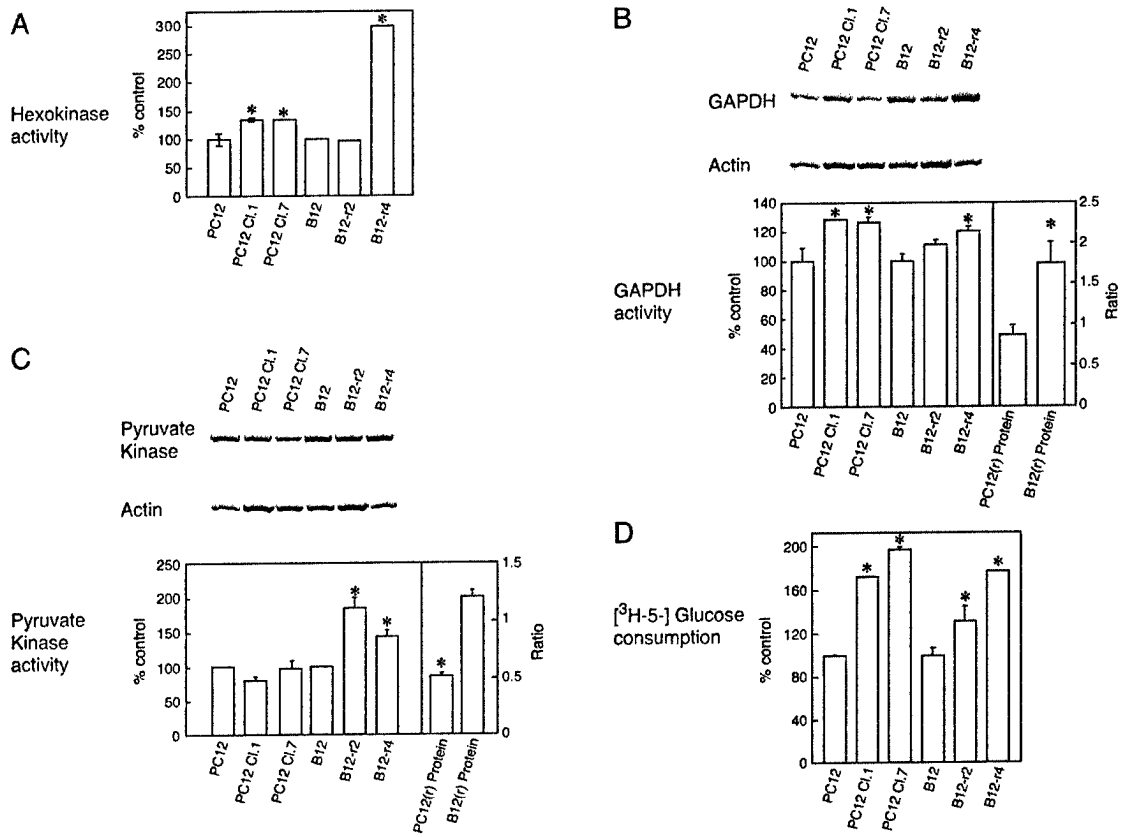

Figure 1. Glycolysis Is Upregulated in Aβ-Resistant Cells

(A) Hexokinase activity. Enzyme activity was measured in cell lysates and is expressed as percent control. Basal hexokinase activity for B12 was 91 ± 6 nmol/min/mg and PC12 71 ± 5.

(B) GAPDH activity and protein expression. GAPDH activity was measured in cell lysates and is expressed as percent control. Enzyme protein and actin in the lysates were determined by Western blotting, and the ratio of enzyme protein to actin is presented in the right panel. The parental line enzyme to actin ratio is set at 100%. There was no change in PC12 enzyme protein, but there was a significant increase in B12 enzyme protein in the two resistant lines. The basal GAPDH activity in PC12 was 135 ± 9 and B12 172 ± 11.

(C) Pyruvate kinase activity and enzyme protein. The data are presented as in (B). The parental enzyme activity for PC12 was 485 ± 21 and for B12 679.

(D) Cells were labeled with ^{3}H-5-glucose for 3 hr, and the production of ^3H_2O determined and presented as percent control (100%). The basal rates of ^3H_2O produced by the parental cells were 5.3 × 10^6 cpm/hr/10^6 cells for B12 and 4.1 × 10^5 cpm/hr/10^6 cells for PC12. All data are the mean of at least three experiments plus or minus the standard error of the mean (SEM). Significantly different from control *$p < 0.01$.

to pyruvate, is, in conjunction with the citric acid cycle, the primary source of reduced NADH. To determine if this pathway is upregulated in Aβ-resistant cells, we analyzed two sets of cells for the expression and activity of several key glycolytic enzymes as well as for the rate of glucose utilization. Three glycolytic enzymes were studied in wild-type and two Aβ-resistant clones of both PC12 and B12. Hexokinase, the irreversible first enzyme in the glycolytic pathway, phosphorylates glucose to glucose-6-phosphate (G6P). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) phosphorylates glyceraldehyde-3-phosphate and generates NADH, while pyruvate kinase, a major regulatory enzyme in the pathway, dephosphorylates phosphoenolpyruvate to generate ATP and pyruvate. Pyruvate is, in turn, the entry point into the citric acid cycle. Hexokinase activity is increased in both of the Aβ-resistant clones of PC12, but in only one clone of the two resistant clones of B12 (Figure 1A). Figure 1B shows that the enzymatic activity of GAPDH is elevated in three of the four Aβ-resistant clones relative to their parental cells. The amount of enzyme protein is also increased in the B12-resistant cells. Finally, we observed a significant increase in pyruvate kinase activity in both of the B12-resistant cell lines but no change in this enzyme in the Aβ-resistant PC12 cells (Figure 1C). There is a decrease in PC12 pyruvate kinase protein, while there is an increase in B12 pyruvate kinase protein. The observation that the activities of all three enzymes are not increased in each resistant cell line is consistent with the fact that individual glycolytic enzymes may initially be in excess in some cells (Esmann, 1978). While these data suggest that glycolysis may be upregulated in the Aβ-resistant cells, the critical factor is the rate of glucose metabolism. We performed two assays to measure this parameter. The first measures the breakdown of ^{3}H-5 glucose to ^3H_2O and the second the conversion of ^{14}C-6-glucose to $^{14}CO_2$. Because neurons can use exogenous amino acids as an alternative energy source to glucose (Honegger et al., 2002), all experiments were done in complete culture

Table 1. Hexose Monophosphate Shunt Activities

	G6PDH (nmol/min/mg)	$^{14}CO_2$ from ^{14}C-1-Glucose (nmol CO_2/hr/10^7 Cells)	$^{14}CO_2$ from ^{14}C-6-Glucose (nmol CO_2/hr/10^7 Cells)	HMS (nmol CO_2/hr/10^6 Cells)	NADPH (pmol/mg)
PC12	46 ± 5	8.4 ± 0.4	3.3 ± 0.5	5.1	105 ± 11
PC12r1	87 ± 7 (1.9)	33.8 ± 0.9 (4.1)	9.6 ± 1.6 (2.9)	24.2 (4.7)	240 ± 31 (2.3)
PC12r7	73 ± 6 (1.6)	17.0 ± 1.2 (2.0)	12.3 ± 2.3 (3.7)	4.7	222 ± 17 (2.1)
B12	975 ± 17	345 ± 51	75 ± 5	270	350 ± 25
B12r2	901 ± 40	422 ± 30	135 ± 13 (1.8)	287	406 ± 44
B12r4	922 ± 19	449 ± 42	147 ± 7 (1.9)	302	375 ± 12
PC12	48 ± 4	6.71 ± 1.0	1.4 ± 0.2	5.2	120 ± 10
PC12 + Aβ	90 ± 5 (1.8)	12.0 ± 0.3 (1.8)	1.7 ± 0.1 (1.2)	10.3 (2.0)	175 ± 8 (1.5)
B12	835 ± 51	295 ± 27	47 ± 3	248	450 ± 35
B12 + Aβ	972 ± 28	301 ± 40	62 ± 4 (1.3)	239	433 ± 60
Primary nerve	20 ± 1	17 ±4	1.5 ± 0.3	15.5	330 ± 55
Primary nerve + Aβ	84 ± 10 (4.2)	51 ± 6 (3.0)	3.8 ± 0.4 (2.5)	47.2 (3.0)	625 ± 72 (1.9)
Primary glia	108 ± 16	96 ± 3	9.2 ± 0.5	86.8	310 ± 15
Primary glia + Aβ	129 ± 11	113 ± 10	6.5 ± 0.2 (0.71)	106.8 (1.2)	412 ± 33 (1.3)

The activities of G6PDH, the molar amounts of NADPH, and glucose metabolism were measured as described in Experimental Procedures. In some experiments, $A\beta_{1-42}$ was added at 2 μM to cultures of PC12, B12, glia, and cortical neurons, and 4 days later glucose utilization, NADPH, and enzyme activity were measured. The results are the means of six determinations ± SEM. Values in parentheses are ratios which are significantly different from controls ($p \leq 0.05$).

medium containing 25 mM glucose. ^{3}H-5 is released in water during the conversion of 2-phosphoglycerate to phosphoenolpyruvate by enolase. Figure 1D shows that the rate of glucose metabolism as measured by this assay is significantly elevated in all of the Aβ-resistant cells. Another assay for glucose metabolism through the glycolytic pathway to the citric acid cycle is the measurement of ^{14}C-6-glucose to $^{14}CO_2$ produced at the α-ketoglutarate dehydrogenase complex. Table 1 shows that the flux of ^{14}C-6-glucose to $^{14}CO_2$ is increased 2- to 3-fold in the four Aβ-resistant cell lines. Therefore, both the overall rate of glycolysis as well as the activity of some of the major enzymes of the glycolytic pathway are elevated in Aβ-resistant cell lines.

Glycolytic Enzymes Are Elevated in AD Brain

It is frequently assumed that when AD brain tissue is examined, one is measuring the physiological parameters of dying cells. However, the reverse is a more likely scenario, as the cells that are still biochemically active (alive) are those that have survived the insults that caused the death of their neighbors. Surviving cells may constitute a resistant population which is analogous to cells selected in vitro for growth in the presence of toxic concentrations of Aβ, and it is very likely that they far outnumber the cells which are in the process of dying. Therefore, if the results with the Aβ-resistant cell lines are related to the Aβ response in AD brain, then it would be expected that glycolytic enzymes would also be elevated in this tissue and in transgenic mice with AD pathology. Figure 2 shows that this is indeed the case. GAPDH enzymatic activity is significantly elevated in lysates from six individual AD brains relative to age-matched controls, as is the mean of the six Alzheimer's GAPDH samples (993 ± 225 nmol/min/mg) versus the mean of controls (570 ± 75 nmol/min/mg) (Figure 2A, right panel). Pyruvate kinase activity and hexokinase activity (Figures 2B and 2C) are also elevated in most of the AD cases relative to age-matched controls.

The Hexose Monophosphate Shunt Is Also Activated

While the conversion of G6P to pyruvate produces NADH during glycolysis, the oxidation of G6P to CO_2 and ribose-5-phosphate produces NADPH via the HMS. Since NADPH is a cofactor required for the maintenance of GSH and other antioxidant activities during conditions of stress, we asked if the HMS is activated in the Aβ-resistant cells and in AD brain. Two assays were used to examine this possibility. Glucose-6-phosphate dehydrogenase (G6PDH) is the first and rate-limiting enzyme in the shunt, and its activity is regulated by oxidative stress (Salvemini et al., 1999). Another assay, which distinguishes HMS activity from glycolysis, is the measurement of $^{14}CO_2$ production from ^{14}C-1-glucose and ^{14}C-6-glucose. ^{14}C-1-glucose releases $^{14}CO_2$ both from the citric acid cycle and via the shunt by the oxidative decarboxylation of 6-phosphogluconate to form ribose-5-phosphate. ^{14}C-6-phosphate releases $^{14}CO_2$ from the citric acid cycle only. HMS activity is determined by the subtraction of $^{14}CO_2$ derived from ^{14}C-6-glucose from $^{14}CO_2$ derived from ^{14}C-1-glucose. Table 1 shows that the HMS is more active in the PC12 Aβ-resistant cell lines than in the parental cell line both in terms of G6PDH activity and in the production of $^{14}CO_2$. However, there is no increased shunt activity in the Aβ-resistant B12 cells relative to controls. This is probably because of the very high basal level of HMS activity in the parental B12 cells. In both cell models, the HMS is more active than the glycolytic pathways. These changes are reflected in an increase in the NADPH levels in the Aβ-resistant PC12 cell lines as compared to the parental line and a high initial amount of NADPH in the B12 parental cell line (Table 1). While it is not possible to measure flux through the glucose degradation pathways in frozen autopsied AD brain tissue, the activity of G6PDH in tissue lysates should reflect the HMS activity of the AD brain. Figure 2D shows that both G6PDH activity and enzyme levels are elevated in four of the six AD brain versus controls and when all AD brains are compared to all controls (right panel). Therefore, as with the glycolytic

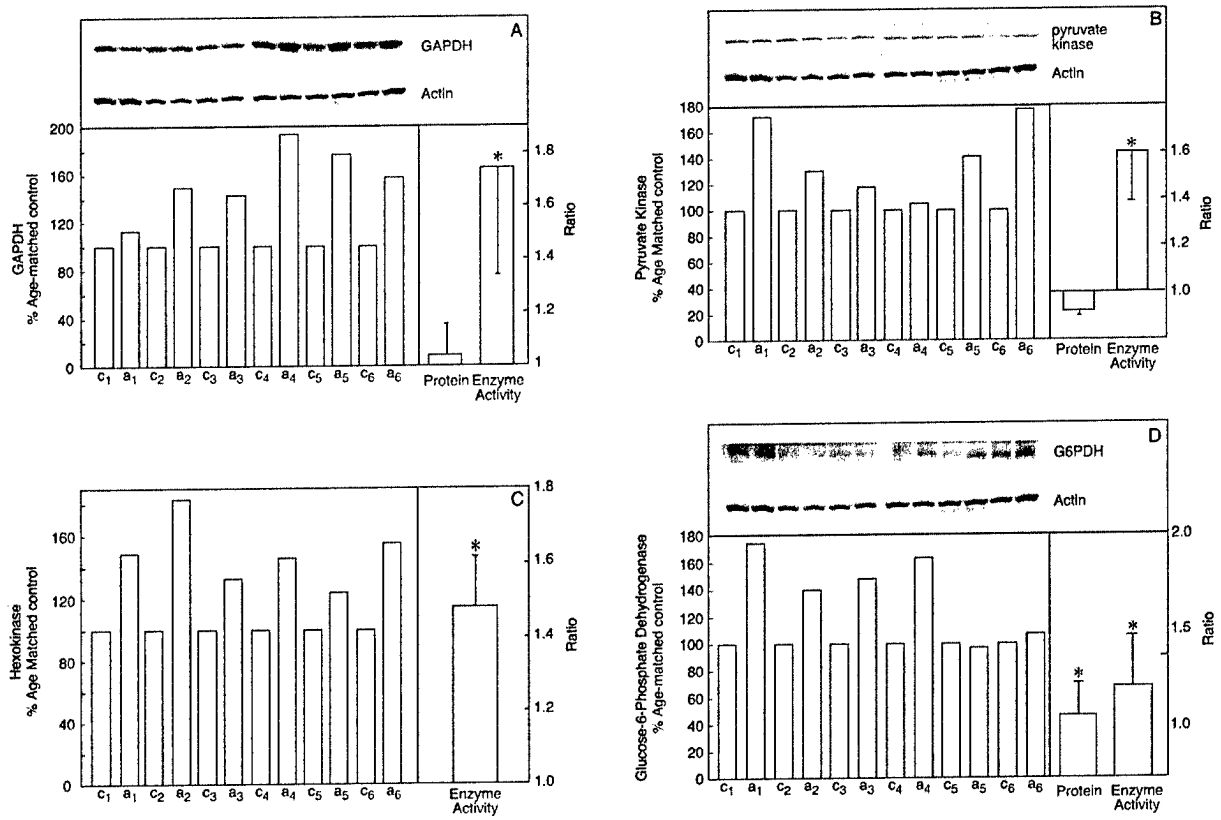

Figure 2. Enzyme Activities in AD Brains

The ratio of enzyme to actin was determined for each sample and then the enzyme/actin ratio in the AD brain sample was divided by the same ratio in the control brain. The lower right sections (A, B, and D) show this ratio; there were no significant differences between control and AD brain in enzyme amount in (A and B), but there is a significant change in G6PDH. The mean increase in the enzymatic specific activity relative to age-matched controls for all six AD cases is also shown. The mean enzyme specific activities for all six AD or all six controls are as follows: GAPDH control, 570 ± 75; GAPDH AD, 993 ± 225; pyruvate kinase control, 729 ± 70; pyruvate kinase AD, 1164 ± 232; hexokinase control 63 ± 5.1; hexokinase AD 93 ± 7.0. G6PDH control 12.45 ± 2.1; and G6PDH AD 17.1 ± 2.4. All experiments were repeated at least three times with similar results. Significantly different from control, *$p \leq 0.05$. "a" is AD brain and "c" is age-matched control brain.

pathway, the rate-limiting HMS enzyme is elevated in AD brain as well as in clonal Aβ-resistant neuronal cells.

To determine if the results observed with HMS and glycolytic enzymes in AD brain could be reproduced in AD mice, we examined samples of the piriform cortex of very old (22 months) Tg2576 mice (Lim et al., 2001) and age-matched controls. Like the AD samples, the transgenic mice have a large increase in G6PDH activity (17.9 ± 8.0 nmol/min/mg, n = 6 versus 6.0 ± 1.8 nmol/min/mg, n = 6, for controls, $p < 0.01$). In addition, there are slight increases in hexokinase and pyruvate kinase, but no change in GAPDH (Table 2). These results show that the upregulation of major components of the glycolytic pathway occurs in AD brain and AD mice as well as in Aβ-resistant cell lines.

Aβ Exposure Directly Enhances HMS Activity and Glucose Uptake

Because oxidative stress can enhance the flux of glucose through the HMS by as much as 100-fold (Salvemini et al., 1999), it is possible that the short-term exposure of cells to the prooxidant Aβ will also enhance this pathway. To examine this possibility, the parental B12 and PC12 cell lines as well as primary rat cortical neurons and astrocyte cultures were exposed to 2 μM of $A\beta_{1-42}$, and the activity of the HMS assayed 4 days later. $A\beta_{1-42}$ killed 10%–15% of the primary nerve cells, suggesting that the surviving population is somewhat resistant. Table 1 shows that $A\beta_{1-42}$ activated the HMS in cortical neurons to about the same extent as glycolysis. The amount of NADPH was also increased in the presence of 2 μM $A\beta_{1-42}$ (Table 1). When similar experiments were done with the parental B12 and PC12 cell lines, Aβ stimulated the shunt activity in PC12 cells, but not significantly in B12 cells, while glycolysis was weakly activated in both. The increased glycolytic activity following Aβ exposure was confirmed by the demonstration that the activity of several of the glycolytic enzymes as well as the conversion of ^3H-5-glucose to ^3H_2O through the glycolytic pathway is activated in cells exposed to Aβ for 4 days (Table 2; Figure 5K).

Although nerve cells have been the primary focus in AD, glia are the majority cell type in the brain. We therefore asked if exposure to Aβ had any effect on glucose metabolism in glia. Tables 1 and 2 show that primary rat cortical astrocytes exposed to Aβ identically to the cortical neurons have a small but significant increase in HMS activities, but that there is a decrease in glycolysis

Table 2. Glycolysis and Glucose Uptake

	^3H_2O from 5-^{3}H-Glucose (pm/hr/10^6 Cells)	Hexokinase (nmol/min/mg)	Pyruvate kinase (nmol/min/mg)	GADPH (nmol/min/mg)	^{14}C-Deoxy-Glucose Uptake (nmol/min/10^6 Cells)
B12	3.2×10^6	111 ± 10	432 ± 31	151 ± 8	33 ± 1.5
B12 + Aβ	5.1×10^6 (1.6)	146 ± 8 (1.3)	693 ± 50 (1.6)	181 ± 10	41 ± 2.8 (1.3)
PC12	5.8×10^5	59 ± 7	420 ± 11	113 ± 5	4.8 ± 0.7
PC12 + Aβ	9.5×10^5 (1.6)	82 ± 10	688 ± 51 (1.6)	117 ± 12	9.7 ± 0.5 (2.0)
Nerve	1.2×10^5	175 ± 11	460 ± 22	1020 ± 74	3.6 ± 0.8
Nerve + Aβ	2.1×10^5 (1.7)	284 ± 18 (1.6)	736 ± 47 (1.6)	1225 ± 85 (1.2)	7.1 ± 1.3 (2.0)
Glia	4.5×10^5	537 ± 22	1780 ± 186	2470 ± 51	7.8 ± 1.2
Glia + Aβ	3.2×10^5 (0.71)	595 ± 17	2571 ± 93	2280 ± 175	4.3 ± 1.2 (0.6)
Control mice	–	40 ± 6	892 ± 65	398 ± 42	–
Tg2576 mice	–	52 ± 4 (1.3)	1128 ± 41 (1.3)	337 ± 23	–
B12 + MIM	6.7×10^6 (2.1)	133 ± 5 (1.2)	820 ± 55 (1.9)	211 ± 30 (1.4)	66 ± 3 (2.0)
PC12 + MIM	11.0×10^5 (1.9)	100 ± 6 (1.7)	630 ± 32 (1.5)	180 ± 21 (1.6)	6.3 ± 0.5 (1.3)
Nerve + MIM	1.7×10^5 (1.4)	225 ± 31 (1.4)	787 ± 80 (1.7)	1140 ± 110	6.5 ± 0.4 (1.8)
B12Z	4.6×10^6	132 ± 10	520 ± 31	138 ± 12	29 ± 4
B12 ZH4	8.3×10^6 (1.8)	211 ± 38 (1.6)	780 ± 70 (1.5)	207 ± 5 (1.5)	46 ± 3 (1.6)
B12 ZH8	7.8×10^6 (1.7)	290 ± 20 (2.2)	884 ± 27 (1.7)	166 ± 15 (1.2)	38 ± 6 (1.3)

The conversion of [5^{3}H]glucose to ^3H_2O, the uptake of ^{14}C-deoxyglucose, and the determination of enzymatic activities were done as described in Experimental Procedures. 2 μM $A\beta_{1-42}$ was added to cultures 4 days before assaying the above activities. Either 100 μM (B12 and PC12) or 50 μM (primary nerve) MIM were added 24 hr before the assays. The uptake of ^{14}C-deoxyglucose was done in 1.6 mM deoxyglucose. The results with the cell lines are the mean of three determinations ± SEM. The data of the AD (Tg) mice represent 4 enzyme assays from each of 6 individual animals in each group (24 assays total for each enzyme). Increases or decreases in parentheses are significant ($p \leq 0.05$).

(Table 2). The glial cells are, however, initially several fold more active in terms of overall glucose metabolism than their neuronal counterparts. An additional parameter relevant to glucose metabolism is the relative rate of glucose uptake. When the rate of ^{3}H-deoxyglucose uptake is examined, there are significant increases in B12 and PC12 Aβ-resistant clones (Figures 3A and 3B) and in cortical neurons exposed to amyloid (Table 2). In contrast, there is a decrease in glial cell deoxyglucose uptake following a 4 day exposure to 2 μM $A\beta_{1-42}$ (Table 2). What are the biological consequences of this Aβ-induced change in nerve glucose metabolism?

Aβ Resistance Leads to Increased Sensitivity to Glucose Starvation

The nervous system has an extremely high rate of energy consumption and glucose is its primary source of energy. It follows that any additional increase in energy burden could lead to a concomitant increase in sensitivity to lower glucose levels. To examine this possibility, parental and Aβ-resistant B12 and PC12 cells were exposed to normal growth medium containing decreased amounts of glucose, and cell viability was measured 2 days later. Figures 4A and 4B show that the Aβ-resistant cells die more readily at higher concentrations of glucose than their parental cell lines. In support of the argument that higher metabolic rates lead to greater sensitivity to glucose starvation, B12 cells, which have a higher metabolic rate than PC12 (Tables 1 and 2), die at a 2-fold higher concentration of glucose than PC12 cells. To rule out the possibility that Aβ resistance leads to an overall increased sensitivity to toxic insults, cells were treated with increasing concentrations of thapsigargin, a potent inducer of the endoplasmic reticulum (ER) stress response (Wong et al., 1993). Figure 4C shows that Aβ-resistant cells were, in contrast to glucose starvation, more resistant to thapsigargin than the parental lines. To determine if Aβ-enhanced glucose sensitivity could be reproduced in cortical neurons, we exposed primary cultures of rat cortical neurons for 4

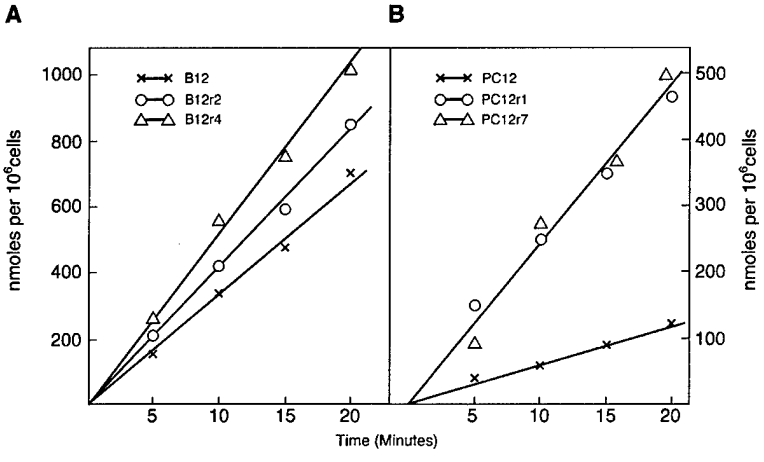

Figure 3. Glucose Uptake in Aβ-Resistant Cell Lines

The uptake of ^{14}C-U-deoxyglucose into B12 (A), PC12 (B), and their resistant clones was assayed in the absence of Aβ. The concentration of exogenous deoxyglucose was 1.6 mM. The experiments were repeated at least three times with similar results.

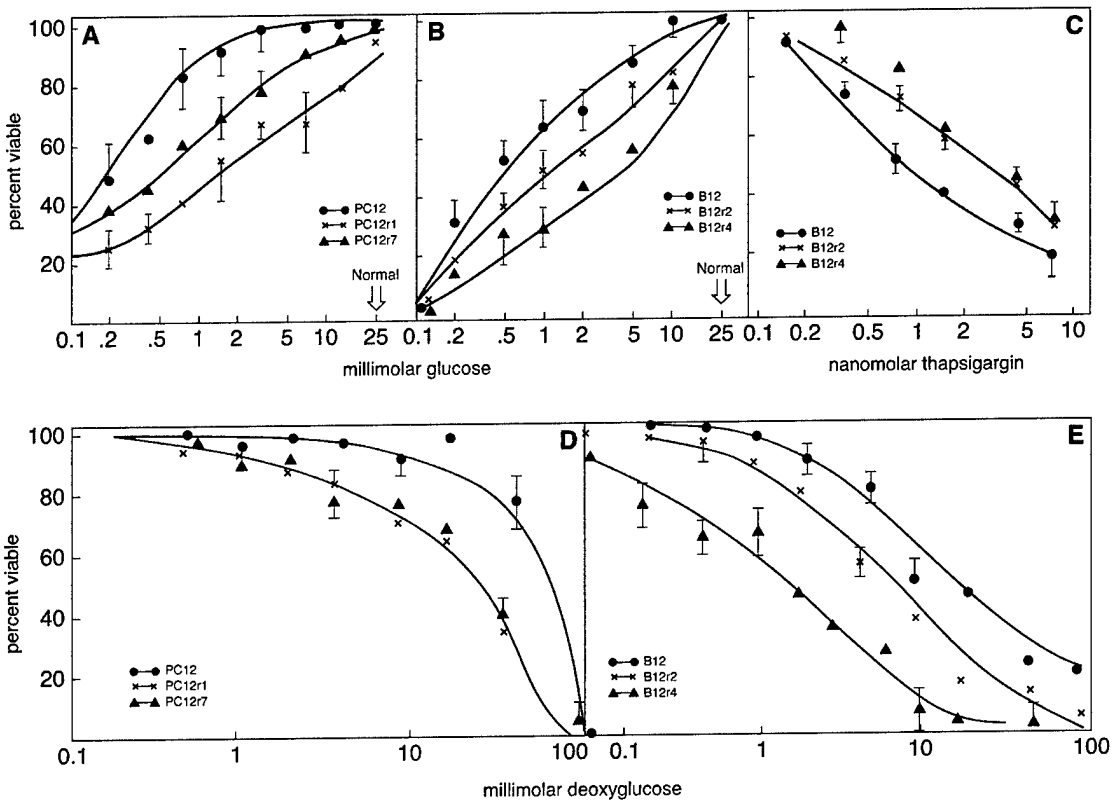

Figure 4. Glucose Starvation of Aβ-Resistant Cells

Exponentially dividing cells of PC12 (A) or B12 (B) were plated in normal growth medium in 96-well plates at 5×10^3 cells per well in the absence of Aβ. Twenty hours later the medium was replaced with a serial dilution of normal (25 mM) glucose containing medium into glucose-free medium, and viability determined 3 days later (C). B12 and its two Aβ-resistant clones were exposed to increasing concentrations of the ER stress inducer thapsigargin and cell viability measured 48 hr later. In a similar experiment to (A), cells were grown in complete medium in the presence of increasing concentrations of deoxyglucose (D and E). Three days later cell viability was determined by the calcein AM assay. The data are presented as the means of triplicate determinations ± SEM. The experiment was repeated at least three times. Using a Wilcoxon signed ranks test for two related samples, $p < 0.01$ for the resistant versus parental cells in all cases.

days to a concentration of $A\beta_{1-42}$, which kills about 10% of the nerve population. Under these conditions, there is an upregulation of the HMS and glycolysis (Tables 1 and 2). The surviving neurons were examined for their sensitivity to glucose starvation. After 4 or 8 days in 2 μM $A\beta_{1-42}$, the treated cells were significantly more sensitive to glucose starvation than the untreated cells (Figures 5A–5C, 5E, 5F, and 5J, quantitated in 5L). There was no change in cell viability when a similar experiment was done with astrocytes.

Deoxyglucose is taken up by cells via the glucose transport system, phosphorylated by hexokinase, but cannot be further metabolized, thereby effectively shutting down glycolysis and the HMS. If a higher rate of glycolysis is responsible for cell death following glucose deprivation, then it would be predicted that the exposure of cells to increasing concentrations of deoxyglucose would kill Aβ-resistant cells more readily than the parental group. Figures 4D and 4E show that when B12 and PC12 cells are grown in normal culture medium and exposed to 0.1–100 mM deoxyglucose, the resistant cells are more sensitive to deoxyglucose than the parental cell lines. Similar results were obtained with primary cortical neurons; the data for 50 μM deoxyglucose are shown in Figures 5D and 5I. These results reveal that Aβ exposure produces a phenotype in which cells die more readily in higher minimal concentrations of exogenous glucose than cells not exposed to amyloid.

Aβ-Resistant Cells Are Also More Sensitive to Antipsychotic Drugs

Glucose is transported into cells by a family of sodium-independent transporters, and in neurons most of the transport is through GLUT3 (Maher et al., 1991). It has been shown that some antipsychotic drugs inhibit GLUT3-dependent glucose uptake in nerve cells (Ardizzone et al., 2001). These data may explain earlier observations that antipsychotic drugs can cause hyperglycemia and diabetes in psychiatric patients (Thonnard-Neumann, 1968). Since Aβ resistance makes nerve cells more sensitive to glucose starvation, it would be predicted that the resistant cell lines would also be more sensitive to these antipsychotic drugs. Figure 5M shows that this is indeed the case, for a concentration of clozapine or fluphenazine that kills only 20% of the parental PC12 cells kills over 70% of the Aβ-resistant PC12 cells. Fluphenazine was also more toxic to B12 Aβ-resistant cells than the parental cells, while the major metabolite of clozapine, clozapine-N-oxide, which is inactive in blocking glucose uptake, is nontoxic. When we repeated

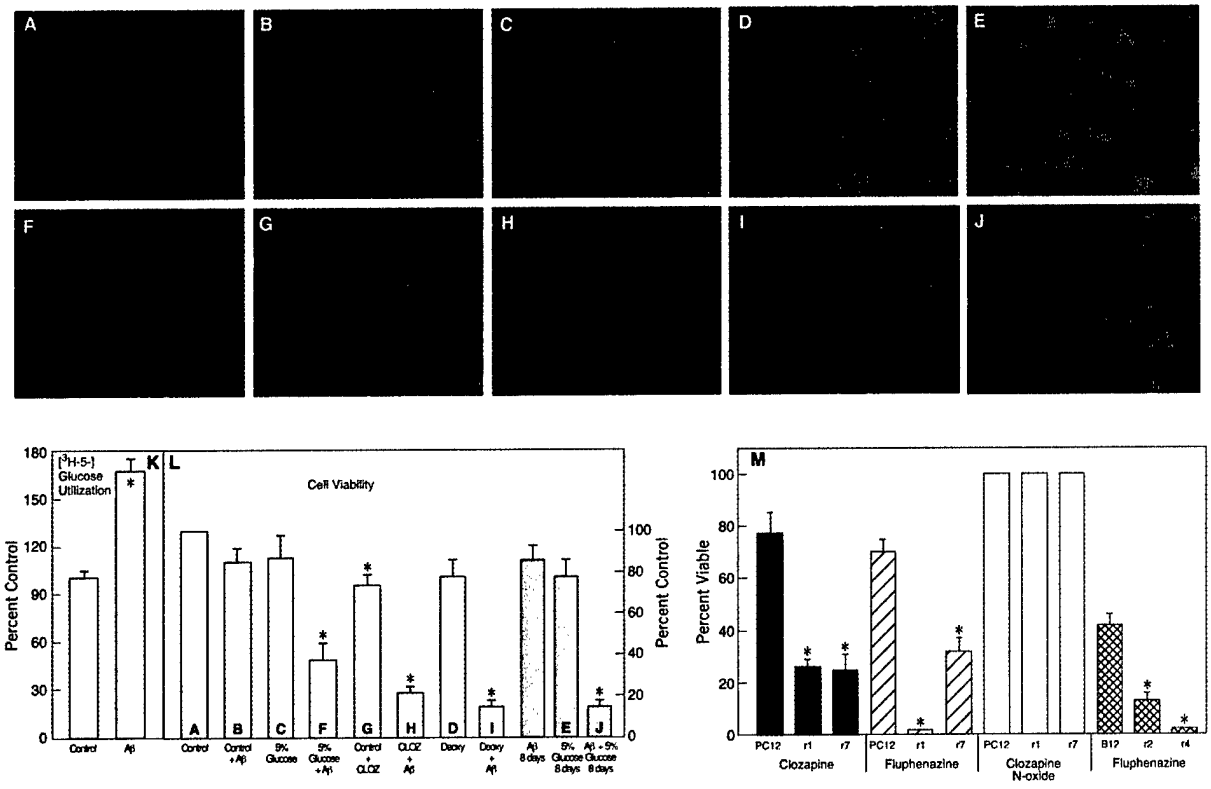

Figure 5. Neurons Exposed to Aβ Are More Sensitive to Glucose Starvation and Antipsychotic Drugs

Cortical neurons were exposed to 2 μM $A\beta_{1-42}$ for 4 or 8 days where indicated. Cells were then placed in fresh medium containing the indicated amounts of glucose, deoxyglucose, or drugs, and 2 days later cell viability was assayed by calcein AM viability stain. The conversion of [5-^{3}H]glucose to ^3H_2O was also monitored (K). (A) Control. (B) $A\beta_{1-42}$. (C) 5% normal glucose (1.25 mm). (D) 50 μM deoxyglucose. (E) 8 days, 5% glucose, note cells more clumped. (F) 5% glucose plus Aβ. (G) Control plus 20 μM clozapine. (H) $A\beta_{1-42}$ plus clozapine. (I) 50 μM deoxyglucose plus Aβ. (J) Eight days, 5% glucose plus Aβ. The data are quantified in (L) as the mean of four determinations ± SEM. Statistically different from control, *$p < 0.01$. (M) Increasing amounts of clozapine, fluphenazine, and clozapine N-oxide (0–100 μM) were added to cultures of PC12, B12, and their Aβ-resistant derivatives. Two days later cell viability was determined by the calcein AM assay. The data for 20 μM drug are shown. The data are the means of triplicate determinations ± SEM. Significant difference from control, *$p \leq 0.01$.

these experiments with primary cortical neurons exposed to marginally toxic concentrations of Aβ, similar results were obtained. Figures 5G, 5H, and 5L show that while 2 μM $A\beta_{1-42}$ or 20 μM clozapine were relatively nontoxic alone, when combined, the majority of the cells in the cortical population died. These results show that there can be a toxic interaction between some antipsychotic drugs and Aβ-resistant cells. Given the profound effects of Aβ on glucose metabolism and cell physiology, what is the mechanism by which Aβ mediates this effect?

Aβ Induces HIF-1

The above data show that glucose metabolism is upregulated by Aβ. Earlier studies showed that metal chelators protect cells from Aβ toxicity (Schubert and Chevion, 1995). Since iron chelators activate the transcription factor HIF-1, which, in turn, increases the transcription of genes for the glycolytic pathway and glucose uptake, it was asked if HIF-1 is responsible for the changes associated with Aβ resistance. This issue was addressed in five ways. It is shown that HIF-1α protein is stabilized in Aβ-resistant cell lines, that Aβ directly induces HIF-1 DNA binding, and that metal chelators and the stable expression of a nondegradable form of HIF-1α reproduce the physiological consequences of Aβ resistance and lower ROS accumulation in cells. Although the highly protease sensitive HIF-1α protein is degraded in our post-mortem AD brain samples, an elevated expression of HIF-1α was observed in AD transgenic mice.

Two assays were used to measure HIF-1 activation, electrophoretic mobility shift assays (EMSAs), and the appearance of HIF-1α protein by Western blotting. Figure 6A shows that both HIF-1 binding to DNA and HIF-1α protein are increased in the Aβ-resistant clones of PC12 and B12 relative to the parental clones. There is a higher basal expression of HIF-1 activity in wild-type B12 cells, perhaps reflected in their high basal glucose metabolism (Table 2). When PC12, B12, and primary cultures of rat cortical neurons are exposed to concentrations of Aβ sufficient to enhance glycolysis, there is an induction of HIF-1 activity as measured in an EMSA time course (Figure 6B), and an increase in HIF-1α protein (Figure 6C). We were unable to detect HIF-1 DNA binding in the cortical neuron cultures, presumably because cortical neurons make different isoforms of HIF-1α which poorly recognize the response element in the

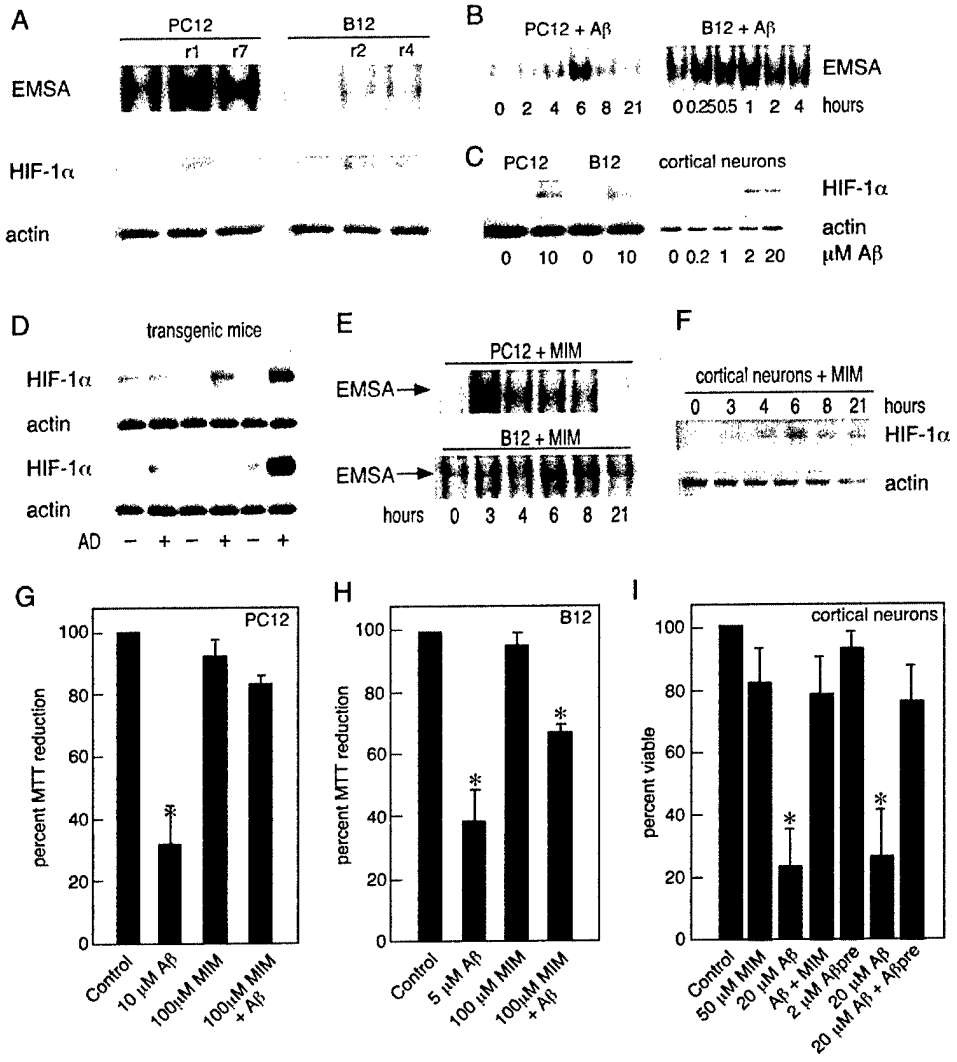

Figure 6. HIF-1 Activity and Aβ

(A) PC12 wild-type and Aβ-resistant clones r1 and r7, and B12 wild-type and Aβ-resistant clones r2 and r4 were assayed for HIF-1 activity by EMSA and Western blotting.

(B) Cells were exposed to 10 μM Aβ and EMSAs done at the indicated times.

(C) Left panel: Western blots of HIF-1α following exposure of PC12 and B12 to 10 μM Aβ (10) or solvent (0). Actin was a loading control. Right panel: Western blot of primary cortical neurons with anti-HIF-1α 6 hr following exposure to the indicated concentration of $Aβ_{1-42}$.

(D) Cortical extracts from six 22- and 23-month-old Tg2576 mice and six age-matched controls were run on SDS acrylamide gels and blotted with anti-HIF-1α or anti-actin. −, control; +, Tg2576.

(E) Cells were exposed to 100 μM MIM and EMSAs done at the indicated times.

(F) Cortical neurons were exposed to 50 μM MIM and lysates immunoblotted with anti HIF-1α or actin at the indicated times.

(G and H) PC12 or B12 cells were exposed to the indicated concentration of Aβ and/or MIM and the ability to reduce MTT assayed 20 hr later.

(I) First four bars from left: cortical neurons were exposed to the indicated concentrations of MIM or $Aβ_{1-42}$ and cell viability assayed by calcein AM 2 days later. Last three bars on right: cortical cells were either exposed to 2 μM Aβ for 4 days and then exposed to 20 μM $Aβ_{1-42}$ for 2 days (20 μM Aβ + Aβ pre) or no additional Aβ (2 μM Aβ pre). Some cells were exposed at 4 days directly to 20 μM Aβ without preexposure (20 μM Aβ). Significantly different from controls, *$p < 0.01$.

EPO enhancer (Drutel et al., 2000). There is, however, a clear accumulation of HIF-1α in the cortical cells following Aβ exposure that is detectable at 2 μM $Aβ_{1-42}$ (Figure 6C). To determine if the cell culture results could be reproduced in vivo, 22-month-old AD transgenic mouse cortex was compared to age-matched controls. Figure 6D shows that five of the six AD mice had elevated HIF-1α expression compared to control animals.

HIF-1 Activation Is Sufficient for Neuroprotection

To determine if the induction of HIF-1 activity is sufficient to cause resistance to Aβ, HIF-1 activity was induced by the metal chelator mimosine (MIM) or by the expression of a nondegradable form of HIF-1α. It was then asked if cells are protected from Aβ toxicity. When PC12 and B12 cells were exposed to MIM, both show an increase in HIF-1 DNA binding (Figure 6E). Figures 6G

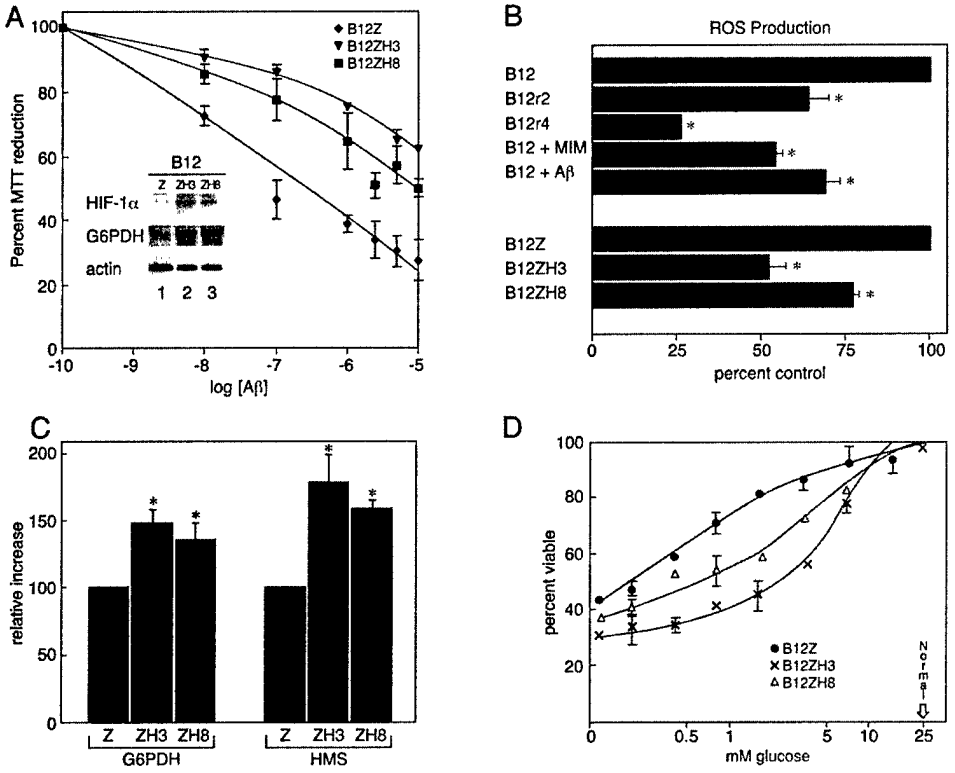

Figure 7. Constitutive HIF-1α Expression

B12 cells were transfected with a nondegradable form of HIF-1α and assayed for several phenotypic changes.

(A) B12 cells permanently transfected with empty vector (B12Z, insert), and two mutant HIF-1α transfected clones (B12H3 and B12H8, insert) were exposed to $A\beta_{1-42}$ and their reactions to the peptide monitored by the MTT assay 24 hr later. G6PDH was also assayed by Western blotting in these three cell lines (insert). Closed diamonds, B12Z (empty vector); closed triangles, B12ZH3 (mutant HIF-1α); closed squares, B12H8 (mutant HIF-1α).

(B) B12 and B12 Aβ-resistant cells, B12 cells exposed to 100 μM or 2 μM $A\beta_{1-42}$ for 20 hr, or the mutant HIF-1α transfected cells were assayed for intracellular peroxides (ROS). The ROS data are presented as fraction of control (B12 or B12Z) values.

(C) Either the enzymatic activity of G6PDH (left side) or total activity of the shunt (HMS, right side) were assayed for B12Z(1), B12ZH3(2), and B12ZH8(3).

(D) B12Z (closed circles), B12ZH3 (Xs) or B12ZH8 (open triangles) were plated in decreasing amounts of glucose and cell viability determined 3 days later by the calcein assay. Significantly different from control, *$p \leq 0.01$.

and 6H show that MIM also protects both PC12 and B12 cells from Aβ to toxicity. To determine if primary cultures of rat cortical neurons respond in a similar manner, cultures were exposed to MIM, followed by a toxic concentration of Aβ. MIM increases HIF-1α levels and protects cortical neurons from Aβ toxicity (Figures 6F and 6I). Table 2 shows that MIM enhances glycolysis and glucose uptake in all three sets of cells. Finally, when primary cortical neurons were exposed to a subtoxic dose of $A\beta_{1-42}$ followed 4 days later by a toxic dose, the cells were protected from Aβ to a level similar to MIM protection (Figure 6I).

To ask if the activation of HIF-1 alone can render cells resistant to Aβ, a nondegradable mutant of HIF-1α was made (Srinivas et al., 1999). Clones transfected with the mutated HIF-1α have elevated levels of HIF-1α protein compared to a clone transfected with the empty vector (Figure 7A, insert). When tested for the ability to resist Aβ toxicity, the mutant HIF-1α expressing cell lines demonstrated a higher tolerance to Aβ (Figure 7A). This result shows that HIF-1 alone is able to rescue cells from Aβ toxicity. The expression of the nondegradable form of HIF-1α also increases glycolysis (Table 2).

Aβ Resistance Leads to Reduced ROS

There is frequently a reciprocal relationship between glycolysis and respiration through the mitochondrial electron transport chain (Webster et al., 1990), and enhanced glycolysis caused by HIF-1 activation can lead to the reduction of ROS accumulation (Michiels et al., 2002). Figure 7B shows that in B12 cells resistant to Aβ, or cells exposed to nontoxic levels of Aβ, there is a decrease in intracellular ROS. A similar reduction in ROS is observed in cells in which HIF-1 is activated by MIM or by the overexpression of the nondegradable form of HIF-1α (Figure 7B).

The HMS is more active in Aβ-resistant cells and in cells exposed to low levels of Aβ (Table 1). To determine if HIF-1 is sufficient to induce this pathway, HIF-1 was activated by the expression of the nondegradable HIF-1α and the activity of the shunt monitored. Figure 7C shows that constitutive HIF-1α activated G6PDH en-

zyme activity and HMS activity as determined by monitoring radioactive glucose metabolism. The expression of G6PDH protein is also increased (Figure 7A, insert). Therefore, along with glycolysis, HIF-1α activation promotes HMS activity, generating the potent reducing molecule NADPH.

The Activation of HIF-1 Enhances Sensitivity to Glucose Starvation

Since cells overexpressing HIF-1α have an enhanced rate of glycolysis (Table 2), it follows that they should be more sensitive to glucose starvation than cells transfected with plasmid alone. To test this possibility, cells were plated in normal medium, and the next day the medium was replaced with complete medium containing decreasing amounts of glucose. Cell viability was determined 3 days later. Figure 7D shows that clones of cells expressing the nondegradable HIF-1α were killed more readily than cells containing the plasmid alone. These data show that while HIF-1 expression is protective for Aβ toxicity, its activity also leads to increased sensitivity to glucose starvation.

Discussion

One manifestation of AD is the oxidative damage of proteins, lipids, and nucleic acids in the brain (Smith et al., 1997). To combat this type of stress, cells need to produce large amounts of reducing equivalents to regenerate GSH and to support the function of antioxidant enzymes. There are two major ways of producing these molecules. One is through glycolysis, which produces NADH, and the other is the HMS that generates NADPH. The above data show that the flux of glucose through both pathways is elevated in amyloid-resistant neuronal cell lines and primary cortical nerve cells exposed to sublethal concentrations of $A\beta_{1-42}$. The cells in AD brain, which by definition represent the surviving, more Aβ-resistant population, also increase the net activity of enzymes in both pathways. These results are consistent with those showing an increased level of G6PDH in surviving pyramidal neurons (Russell et al., 1999) and an increase in two enzyme activities associated with the HMS in AD brain (Palmer, 1999). The ability of Aβ to increase glycolysis, HMS activity, and antioxidant defenses may explain the observation that low levels of Aβ are neuroprotective against the prooxidant conditions of tissue culture, while higher concentrations are toxic (Yankner et al., 1990). These changes in AD brain may be a reflection of metabolic changes within the individual cells or they may be due to anatomical changes, such as altered nerve/glia ratios, due to the progression of the disease. In cultured cells, and possibly in AD, the increase in metabolic activity is a consequence of the activation of HIF-1 by subtoxic amounts of Aβ. The conclusion that HIF-1 regulates the Aβ response is based upon the following observations: (1) Aβ-resistant cell lines have elevated HIF-1 expression and activity. (2) An iron chelator that stabilizes HIF-1α and increases HIF-1 binding to DNA induces expression of the same group of enzymatic activities as low concentrations of Aβ, and protects cell lines and primary cortical neurons from Aβ toxicity (Figures 6 and 7; Table 2). (3) The expression of a nondegradable mutant of HIF-1α also protects cells from Aβ (Figure 7A). (4) Exposure of cells to low levels of Aβ induces HIF-1 and protects nerve cells from the toxicity of high Aβ concentrations (Figure 6). (5) The direct activation of HIF-1 by MIM or a nondegradable form of HIF-1α mimics the physiological consequences of nontoxic levels of Aβ (Figure 7; Table 2). (6) HIF-1α is more highly expressed in AD transgenic mice than in age-matched controls (Figure 6D).

Iron chelators protect cultured cells from Aβ (Schubert and Chevion, 1995) and inhibit the progression of AD (McLachlan et al., 1991). Furthermore, treating AD transgenic mice with a metal chelator clioquinol reverses some aspects of AD-like pathology (Cherny et al., 2001) and blocks MPTP-induced Parkinson's pathology (Kaur et al., 2003). Clioquinol induces HIF-1 with an IC_{50} of 600 nM (data not shown). Although the published results were attributed to metal chelators binding to prooxidant metal ions, our data strongly suggest that an important part of their success is due to their ability to induce HIF-1. In contrast to nerve cells, glycolysis is downregulated by Aβ in glial cells (HMS activity is, however, upregulated), even though HIF-1 is activated by Aβ in these cells (data not shown).

The activation of HIF-1 has been associated with both death and antideath pathways (Piret et al., 2002). However, in most cases ROS and prooxidants like arsenite stabilize HIF-1α and induce HIF-1 DNA binding (Duyndam et al., 2001), and hypoxic preconditioning produces tolerance to hypoxic-ischemic brain injury (Bernaudin et al., 2002). Since HIF-1 activation protects nerve cells from both oxidative stress and Aβ toxicity, it is likely that the upregulation of this transcription factor is a general neuroprotective mechanism against oxidative insults. Indeed, the Aβ-resistant cell lines used in this study are resistant to glutamate, cystine starvation, H_2O_2, arsenite, and rotenone (Dargusch and Schubert, 2002), as well as thapsigargin (Figure 4C). HIF-1 activation enhances the rate of glycolysis, as well as the activity of the HMS shunt (Figure 7). Therefore, it is likely that the neuroprotection mechanism is through the enhanced production of NADH and NADPH from glycolysis and the HMS, and an increase in the synthesis of the antioxidant pyruvate via glycolysis. All three molecules would contribute to a reduction in intracellular ROS and produce a more reductive environment to ward off future toxic insults. Stress resistance is relative, and the more resistant state can obviously be overridden by higher concentrations of a prooxidant or more stressful physiological conditions (Dargusch and Schubert, 2002). Since Aβ plaque density is not highly correlated with nerve cell loss or cognitive decline (Terry, 2000), it is possible that at least an early function of Aβ in aging is neuroprotection.

The increased rate of glucose consumption does, however, have some negative physiological consequences in clinical situations where glucose uptake is limiting. These include patients exposed to certain classes of antipsychotic drugs, type II diabetes, and ischemia. Phenothiazine-based antipsychotic drugs such as chlorpromazine induce hyperglycemia and diabetes in some patients (Hiles, 1956). It has more recently been established that a large number of antipsychotic drugs effectively inhibit glucose uptake via competition

for glucose transporters (Dwyer et al., 1999). Since these drugs also cause an increased sensitivity to glucose starvation in Aβ-resistant clonal nerve cells and cortical nerve cells exposed to low levels of $A\beta_{1-42}$ (Figure 5), it follows that the administration of this subset of antipsychotics to preclinical or mild AD patients could lead to the expedited death of cells with increased glucose dependency due to Aβ exposure.

Diabetes mellitus (DM) is a probable risk factor for AD, doubling the likelihood of developing the disease (Ott et al., 1999). Insulin stimulates glucose uptake, and AD patients with reduced insulin levels have significantly lower cognitive skills than individuals with normal insulin levels (Craft et al., 1996). Although only a small percentage of the glucose supply to neurons is delivered by an insulin-dependent mechanism (Schulingkamp et al., 2000), a loss of the insulin-dependent glucose uptake may be sufficient to cause the death of Aβ-resistant cells with a high metabolic rate. In fact, the administration of glucose and insulin together enhances the memory of AD patients, while giving glucose alone does not have this effect (Craft et al., 1996; Manning et al., 1993). Therefore, a lack of insulin-stimulated glucose uptake in the population of Aβ-resistant nerve cells with a high glucose requirement could lead to a more rapid progression of the disease.

The role of glucose metabolism in AD is controversial, but the current dogma is that the use of glucose by nerve cells in the affected brain areas is impaired (Blass, 2001; Gibson, 2002). This conclusion is based primarily upon the use of 2-^{18}fluoro-2 deoxyglucose and PET to measure in situ glucose uptake (Mielke et al., 1991), and the apparent inactivation in post-mortem samples of a few enzymes involved in energy metabolism (Blass, 2001). In contrast to the data suggesting a decrease in glucose metabolism in AD, several earlier studies demonstrated that in viable biopsied AD brain tissue there is a highly significant increase in the rate of CO_2 production from glucose and an increase in O_2 uptake in AD versus control brain tissue (Bowen et al., 1979; Sims et al., 1981, 1983). Other studies examining O_2 uptake in biopsied AD tissue showed that the rate of O_2 consumption in the presence of a mitochondrial uncoupling agent is the same in AD versus control tissue, but under conditions of submaximal activity, which reflect the in vivo situation, AD samples have nearly 2-fold higher rates of O_2 uptake (Sims et al., 1987). It should be noted that the mean age for the biopsied patients in the above studies was less than 60 yrs. Supporting the enzyme activity measurements of G6PDH are immunohistochemical data which show an increase in enzyme amount in surviving neurons within AD brain (Russell et al., 1999). Finally is the observation that cerebral metabolism in young Down's syndrome patients is elevated relative to controls, while it is slightly lower in older patients (Schwartz et al., 1983). Down's syndrome patients always develop the pathological features of AD (Wisniewski et al., 1985). The latter data are all consistent with enhanced glucose metabolism in AD versus control tissue, at least in the early stages of the disease, and agree with our data on both the Aβ-resistant cell lines, transgenic mice, and primary cortical neuron cultures exposed to marginally toxic levels of Aβ.

How are the data showing that glucose flux and HMS enzymatic activities are enhanced in AD brain and in Aβ-resistant cells reconciled with the observations that glucose metabolism is reduced in PET scans? As mentioned above, all of the biopsy studies on energy metabolism were done with relatively young AD patients having an average age of less than 60 years (Bowen et al., 1979; Sims et al., 1981, 1983, 1987). In addition, an initial gain and then loss of G6PDH activity with age in AD patients has also been noted (Palmer, 1999). It is therefore possible that the results on cultured cells reported here reflect an early response of neurons to amyloid that is eventually overridden in older individuals by the cumulative effects of age and toxicity, resulting in a net decline in nerve glucose metabolism. However, some aspects of the Aβ-resistant phenotype must persist in older individuals since G6PDH and glycolytic enzymes are clearly upregulated in our AD patients, whose average age was 86. The net decrease in fluorodeoxyglucose uptake observed by PET could also be a result of changes in vascular blood flow due to Aβ damage (Horwood and Davies, 1994) and the additive effects of nerve cell death, nerve terminal destruction, and Aβ-induced reduction of glial cell glucose uptake (Table 2) (Parpura-Gill et al., 1997).

The global changes in glucose metabolism outlined above are examples of system-wide energy dynamics which may play key roles in both AD and other neurodegenerative diseases (Strohman, 2002). The Aβ-induced changes in glucose metabolism that cause reduced ROS levels in cells are analogous to the changes in energy metabolism that have recently been associated with extended life span in yeast (Lin et al., 2002) and fungi (Dufour et al., 2000), and are likely to reflect a life extension program in response to oxidative stress at the cellular level. However, the biological consequences of a neuroprotective increase in nerve cell glucose utilization may be an increased risk of nerve cell death associated with diabetes, ischemia, and psychoactive drug use.

Experimental Procedures

Cell Culture

The B12 and PC12 clonal cell lines, their Aβ-resistant derivatives, and their culture have been described (Behl et al., 1994; Sagara et al., 1996). Mouse and rat astrocytes were prepared from newborn animals as described (Parpura-Gill et al., 1997). Primary cortical neurons were prepared from day 17 rat embryos (Li et al., 1997).

Cell viability was measured by three assays. Following the experimental protocol, viable cells were assayed by incubation with the fluorescent viable stain calcein AM which is concentrated in living cells (Live Dead Assay; Molecular Probes, Eugene, OR) and the cells scored manually from 35 mM culture dishes. At least ten random fields were scored for each data point. When Aβ was not used, the MTT assay was sometimes used in addition to calcein AM. The MTT assay reflects the toxicity of Aβ, but not necessarily cell lysis by Aβ (Liu et al., 1997). Flow cytometry and Western blotting were done as described (Tan et al., 2001).

AD Brain

All autopsied brain samples were obtained from Drs. Carol Miller and Jenny Tang at the Alzheimer's Disease Research Center (ADRC) in the University of Southern California School of Medicine, Los Angeles, CA. All material was from the same area of the frontal cortex and quick frozen after removal. All AD cases and controls were matched pairwise for age, sex, and in most cases, post-mortem time (PMT). The CERAD criterion for plaque density in the AD samples had a mean of 3.5 ± 1, a low toward moderate value. In

Figure 3, the following were shown: (C1) normal control brain, female, age 94, post-mortem time 4.5 hr; (A1) AD brain, female, age 95, PMT 4.5 hr; (C2) control brain, female, age 87, PMT 6 hr; (A2) AD brain, female, age 86, PMT 6 hr; (C3) control brain, male, age 88, PMT 2 hr; (A3) AD brain, male, age 85, PMT 4.5 hr; (C4) control brain, male, age 69, PMT 8 hr; (A4) AD brain, male, age 75, PMT 2 hr; (C5) control brain, female, age 91, PMT not available; (A5) AD brain, female, age 90, PMT 4.5 hr; (C6) control brain, female, age 86, PMT 4 hr; and (A6) AD brain, male, age 86, PMT 4.5 hr. Brain tissue from AD transgenic mice were obtained from Dr. Giselle Lim at the University of California, Los Angeles (Lim et al., 2001). 22- or 23-month-old Tg2576 mice and age-matched controls were perfused with saline with a protease inhibitor cocktail, and the brain regions were snap frozen in liquid nitrogen and stored at −80°C. The piriform cortex that had visible AD pathology was examined.

Enzyme Assays

Three assays were used for glucose utilization. The first was the formation of ^3H_2O from [5-^{3}H]glucose performed as previously described (Ashcroft et al., 1972). The last two assays involved the production of $^{14}CO_2$ from ^{14}C-glucose. D-[1-^{14}C]glucose and D-[6-^{14}C]glucose (NEN, Boston, MA) were used to measure net glucose utilization via the HMS and the citric acid cycle pathways, respectively, according to published procedures (Hyslop et al., 1988). To examine glucose uptake, cells were washed twice with glucose-free DMEM and incubated in this medium for 1 hr at 37°C. [U-^{14}C]deoxyglucose was then added at 0.5 μCi/ml to a final concentration of 1.6 mM and uptake monitored over 20 min during which the time uptake was linear. Uptake was stopped by washing the cells three times with ice-cold phosphate-buffered saline and dissolving the cells in 0.2 N NaOH for isotopic counting. Uptake was completely inhibited by 10 mM phloretin, establishing the specificity of uptake through a glucose transporter. All enzymes were assayed by the standard optical methods (Worthington, 1947). Frozen brain tissue was homogenized in assay buffers and stored at −80°C; cell cultures were washed two times with phosphate-buffered saline and lysed in the same buffer by sonication. In all cases, care was taken to ensure that the reactions were linear and substrate dependent. NADPH was measured as described (Zhang et al., 2000).

EMSAs

Nuclear extracts were prepared and used for an EMSA specific for HIF-1. A ^{32}P-labeled 24 bp oligonucleotide (5′-GCCCTACGTGCTGC CTCGCATGGC-3′) from the mouse EPO 3′ enhancer was used as probe (Maxwell et al., 1999). The identity of the retarded bands was confirmed by adding antibodies specific for HIF-1α or HIF-1β to the incubation mixture (Santa Cruz Biotechnology, Santa Cruz, CA). A super shift of the retarded bands was observed (data not shown). In addition, cell extracts were incubated with a 100-fold excess of unlabeled wild-type oligo or with an oligo mutated in the HRE region; the wild-type oligo eliminated the retarded band while the mutated oligo did not (data not shown).

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